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# Evaluation of Biological Stains, Inks, and Fluorescent Pigments as Marks for Shrimp

By Edward F. Klima

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# Evaluation of Biological Stains, Inks, and Fluorescent Pigments as Marks for Shrimp[1]

by

EDWARD F. KLIMA, Fishery Biologist (Research)

## ABSTRACT

Studies were made in the laboratory to find marking materials that can be used to distinguish groups of shrimp in mark-recapture experiments designed to estimate rates of population growth and mortality. A series of combination marks, formed by injecting various biological stains and fluorescent pigments, was developed. These marks are permanent and do not affect survival.

## INTRODUCTION

Mark-recapture experiments provide a simple and direct means for estimating growth and mortality in fish and shellfish populations. Recent development of the stain-injection technique of marking has greatly improved our ability to estimate these vital parameters in stocks of penaeid shrimp. Petersen disk tags, which have been used to mark shrimp, may affect survival and growth, whereas marking with stains has no discernible effect.

Until recently, the staining technique has been somewhat limited by the small number of suitable stains available. Of those tested by Dawson (1957) and Costello (1964), only Trypan blue, Niagara sky blue 6B, Trypan red, and fast green FCF were retained permanently in the gills of shrimp. Trypan red is not suitable for use as a marking agent, however, because it does not contrast sufficiently with the shrimp's natural coloration to permit shrimp so marked to be easily identified by fishermen. Shrimp marked with Trypan blue and Niagara sky blue 6B cannot be differentiated from one another and therefore cannot be used to identify groups that might overlap spatially during a particular study. The objective of this investigation was to increase the number of distinguishable marks that can be used simultaneously in mark-recapture experiments.

Accordingly, laboratory tests were undertaken to find additional materials for marking shrimp and to determine the effect of these marks on shrimp survival. In the first few experiments, shrimp were held in groups of 20 or more in large tanks supplied with either fresh or recirculating filtered sea water. In later experiments, they were kept in small individual compartments. The experimental shrimp ranged in total length from 55 to 179 mm.--sizes that would normally be involved in mark-recapture studies. They were caught in Galveston Bay and included the three species fished commercially in the northern Gulf of Mexico, namely, the white, brown, and pink shrimps, Penaeus setiferus, P. aztecus, and P. duorarum, respectively.

## TYPES OF MARKS

Permanent, nonlethal marks that can be easily detected by casual observers are desirable for quantitative mark-recapture studies designed to provide estimates of mortality and growth rates. Marks that do not fulfill all of these qualifications may satisfy the less demanding requirements of studies undertaken to obtain general information on shrimp movements.

### PRIMARY MARKS

Primary marks are formed upon injection of pigments (e.g., fast green FCF, Niagara sky blue 6B, and Trypan blue) that remain in the gills of shrimp and differ sufficiently from the animal's natural color to be easily detected by fishermen. Aqueous solutions of biological stains, commercial food colors, and inks were

[1]Contribution No. 200, Bureau of Commercial Fisheries Biological Laboratory, Galveston, Tex.

tested as primary marks. They were applied by injecting from 0.03 to 0.08 ml. of marking material between the shrimp's fifth and sixth abdominal segments following the method described by Costello (1964). Of the 65 dyes and inks so tested (table 1), none was found to be fully suitable for use in growth and mortality studies according to my definition, and only four were deemed satisfactory for use as marking agents in experiments dealing solely with shrimp movements.

The latter included green and blue Bates[2] machine inks, both of which concentrated in the branchia within 24 hours after injection and could be visually differentiated from fast green and Trypan blue. The green ink produced a vivid mark that could be identified for up to 40 days following application, but thereafter began to fade. The blue ink was not sufficiently striking in color to be distinguishable as a mark by casual observers, but could be detected by trained observers. It also began to fade after about 40 days.

Various combinations of Trypan red and Trypan blue (0.25 percent solutions) were tested as marks. Two of these combinations, 2 to 1 and 5 to 1 Trypan red to Trypan blue, produced marks that were permanent but rather dull and difficult to see. Consequently, shrimp stained with these marks would not be easily detected by fishermen or shrimp processors, and their use would have to be limited to laboratory studies or to field experiments in which commercial catches are examined by trained observers.

## SECONDARY MARKS

A secondary mark is a spot of color used to code and differentiate a primary mark. Such a mark may or may not be apparent to the casual observer, but can be distinguished by careful visual or fluorometric examination. A secondary mark, since it is not necessarily obvious, must be used with a primary mark. Various inks and fluorescent materials were tested as secondary marks (table 2) and were applied together with a primary stain to form a double mark by either the single-injection, double-injection, or tattooing method of marking.

### Double-Injection Method of Marking

The double-injection method consists of two separate injections, first with a primary stain and subsequently with a secondary pigment. An aqueous solution of a primary stain, fast green or Trypan blue, was injected as described previously, and a secondary material was applied by means of a 1- or 2-cc. tuberculin syringe with a 25- or 27-gage needle. From 0.003 to 0.010 ml. of the secondary marking material was injected through the articular membrane of either the sixth abdominal joint to the left of the middorsal line, or to one side of the midventral line between the second and third abdominal segments. In the latter instance, the needle was directed anteriorly just beneath the cuticle of the exoskeleton. The location of primary and secondary marks is illustrated in figure 1.

Inks.--For any ink to be suitable as a secondary mark, most of it must remain at the injection site. The primary mark is obscured if too much ink migrates into the branchial region, thus preventing differentiation of the marked individuals. Red, blue, and black checkwriter inks (Sanford), diluted with castor oil or undiluted, were tested for use as secondary marks. The results from these tests are listed in table 3. In each case, the condition of the secondary mark was recorded at the time of death as good, poor, or unsatisfactory if no mark was apparent. Neither the diluted nor undiluted inks produced wholly satisfactory secondary marks because ink moved from the injection site into the gills and frequently masked the primary marks. Of the inks tested, the undiluted red and blue inks produced the best marks. These two inks are suitable for use as marks in field studies designed to obtain general information on movement.

Fluorescent pigments.--A series of seven laboratory experiments (table 4) was made to determine the suitability of fluorescent pigments for use as secondary marks. The pigments Day-Glo neon red A-12, blaze orange A-15, arc yellow A-16, Saturn yellow A-17, and resoform fluorescent yellow 10-2001 (General Dyestuff) could be readily distinguished from one another. These five pigments were found to provide suitable secondary marks that could be used with primary stains of established reliability. Mixtures of 1.5 to 4.0 percent fluorescent pigment in petroleum jelly produced secondary marks that were detectable under ultraviolet light.

The fluorescent marking material was most easily injected into the experimental animals at temperatures ranging from 70° to 90° F. Lower temperatures increased the viscosity of the petroleum jelly and made it difficult to eject the mixture from the syringe. Conversely, at temperatures greater than 90° F., the viscosity of the petroleum jelly was decreased and more material was often injected into the animals than was necessary for identification purposes.

In contrast to the primary marking materials, the fluorescent pigment usually remained at the injection site, although in some cases minute amounts could be found in the

[2]Trade names referred to in this publication do not imply endorsement of commercial products.

Table 1.--Inks and dyes tested and found to be unsatisfactory for use as primary marks

| Manufacturer and trade name | Concentration of dye | Experimental shrimp | Remarks |
|---|---|---|---|
| Dyes | | | |
| Arthur H. Thomas Co., Philadelphia, Pa.: | Percent | Number | |
| Fluoresceine | 1.0 | 10 | Faded within 1 week. |
| Baltimore Biological, Baltimore, Md.: | | | |
| Brom cresol purple | 1.0 | 10 | Do. |
| Resazurin | 1.0 | 10 | Do. |
| Fisher Scientific, New York, N.Y.: | | | |
| Brom phenol blue | 1.0 | 10 | Do. |
| French Food Co., Rochester, N.Y.: | | | |
| Blue liquid food colors | 100.0 | 10 | Do. |
| Green liquid food colors | 100.0 | 10 | Do. |
| Red liquid food colors | 100.0 | 10 | Do. |
| Yellow liquid food colors | 100.0 | 5 | Do. |
| Harleco, Philadelphia, Pa.: | | | |
| Acid alizarin blue | 1.0 | 15 | Do. |
| Acid violet 10B | 1.0 | 15 | Do. |
| Acridin orange | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Aniline blue | 1.0 | 10 | Faded within 1 week. |
| Blue 2B | 1.0 | 15 | Do. |
| Brilliant alizarin blue | 1.0 | 10 | Do. |
| Brilliant blue FCF | 1.0 | 10 | Do. |
| Brilliant milling green | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Brilliant milling green B | 1.0 | 10 | Faded within 1 week. |
| Brilliant violet | 1.0 | 10 | Do. |
| Brom cresol green WS | 1.0 | 15 | Do. |
| Brom cresol purple WS | 1.0 | 15 | Do. |
| Brom cresol thymo blue WS | 1.0 | 15 | Do. |
| Carmin alum Mayer | 1.0 | 10 | Do. |
| Clayton yellow | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Diamond blue 3-B | 1.0 | 10 | Faded within 1 week. |
| Direct green B | 1.0 | 15 | Do. |
| Eriochrome violet BA | 1.0 | 15 | Do. |
| Fuchsin acid | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Hofman violet | 1.0 | 10 | Faded within 1 week. |
| Jenner stain | 1.0 | 10 | Do. |
| Light green SF | 1.0 | 15 | Do. |
| Malachite green | 1.0 | 10 | Do. |
| Meldola blue | 1.0 | 10 | Do. |
| Naphthol | 1.0 | 10 | Do. |
| Naphthol green B | 1.0 | 15 | Do. |
| Neutral violet | 1.0 | 15 | Do. |
| Phosphine | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Primuline | 1.0 | 10 | Lethal within 1 week. |
| Rhodamin B | 1.0 | 10 | Faded within 1 week. |
| Rhodamin 6B | .5 | 10 | Do. |
| Safarine | 1.0 | 10 | Do. |

Table 1.--Inks and dyes tested and found to be unsatisfactory for use as primary marks--Continued

| Manufacturer and trade name | Concentration of dye | Experimental shrimp | Remarks |
|---|---|---|---|
| | Percent | Number | |
| Solray purple | 1.0 | 10 | Do. |
| Tanus green B | 1.0 | 15 | Do. |
| Thioflavine S | .5 | 20 | Do. |
| Toluidin blue O | 1.0 | 10 | Do. |
| Uranin | 1.0 | 10 | Do. |
| Victoria blue B | 1.0 | 10 | Do. |
| Victoria green | 1.0 | 10 | Do. |
| K&K Laboratories, Inc., Jamaica, N.Y.: | | | |
| Pontamine fast pink | 1.0 | 10 | Faded within 1 week. |
| Rivanal | .5 | 10 | Do. |
| Thionine | 1.0 | 10 | Do. |
| National Aniline, New York, N.Y.: | | | |
| Acriflavin neutral NF | 1.0 | 10 | Lethal within 1 week. |
| Crystal violet | 1.0 | 10 | Faded within 1 week. |
| DuPont dearmour blue | 1.0 | 10 | Lethal within 1 week. |
| Indigo carmine | 1.0 | 10 | Faded within 1 week. |
| Methylene blue chloride | 1.0 | 10 | Do. |
| W. H. Curtin & Co., Houston, Tex.: | | | |
| Methyl violet 2-B | 1.0 | 10 | Do. |
| Rose Bengal | .5 | 10 | Do. |
| INKS | | | |
| Bates Mfg. Co., Orange, N.J.: | | | |
| Purple | 100.0 | 20 | Faded and/or lethal within 1 week. |
| Black | 100.0 | 30 | Lethal within 1 week. |
| Harshaw Chemical Co., Cleveland, Ohio: | | | |
| Millioni blue | 1.0 | 10 | Faded and/or lethal within 1 week. |
| Higgins India Ink Co., Brooklyn, N.Y.: | | | |
| Purple | 100.0 | 10 | Faded and/or lethal within 1 week. |
| Black | 100.0 | 10 | Do. |
| National Cash Register Co., Dayton, Ohio: | | | |
| Volatile blue | 100.0 | 10 | Do. |
| Volatile green | 100.0 | 10 | Do. |
| Volatile purple | 100.0 | 10 | Do. |

Table 2.--Trade names and manufacturers of secondary marking materials

| Manufacturer and trade names | Fluorescent color |
|---|---|
| Inks: | |
| Sanford Ink Co. Bellwood, Ill. | |
| Black checkwriter ink No. 638 | None |
| Blue checkwriter ink No. 635 | Do. |
| Red checkwriter ink No. 638 | Do. |
| Pigments: | |
| General Dyestuff Co. New York, N.Y. | |
| Resoform Fluorescent yellow . 10-2001 | Yellow |
| Switzer Bros., Inc. Cleveland, Ohio | |
| Arc yellow A-16 | Yellow |
| Blaze orange A-15 | Orange |
| Neon red A-12 | Red |
| Saturn yellow A-17 | Yellow |
| Solutions: | |
| Printing Arts Research Laboratory, Santa Barbara, Calif. | |
| Fluorographic | Chalk white |

ventral sinuses or in the gills (table 4). In no instance, however, did these secondary pigments obsucre the primary marks. Both the primary and secondary marks could easily be identified up to 276 days after marking. The marks did not appear to fade, nor were they lost during molting.

A laboratory experiment was conducted to determine whether it would be possible to localize the secondary pigments in more than one area of the shrimp's body in order to increase the number of different marks that could be used simultaneously in a mark-recapture experiment. It was found that the secondary pigments could be confined to the middorsal surface at the joint of the sixth abdominal segment and to the ventral surface between the second and third abdominal segment, thereby effectively doubling the number of distinguishable secondary marks.

The possibility that the fluorescent material may be lethal to shrimp was also investigated. The first few experiments provided only preliminary information because mortality was confounded by cannibalism or escapement of shrimp. However, the results from these experiments suggested that there was no difference in survival between groups of shrimp injected with fast green and any of the five fluorescent marks.

In later experiments, it was therefore deemed necessary to determine the effect on survival of only one of the fluorescent marks used with fast green or Trypan blue. The survival of shrimp in this series of experiments (table 5) was not affected by escapement or cannibalism, because each experimental animal was held in a separate compartment. Each experiment included three groups of shrimp, of which one was unmarked, another was injected with distilled water, and a third was injected with a combination mark. The first group served as a control and was compared to the second group to determine the effect on survival of the treatment associated with marking. The second group, in turn, acted as a control for the third group to ascertain the influence of the combination mark on survival. Mortality was generally most variable during the first few days after marking and thereafter remained relatively constant. Fewer shrimp died in the marked groups than in the control groups; therefore, neither the marking materials nor the method of marking has any lasting effect on survival. The five fluorescent

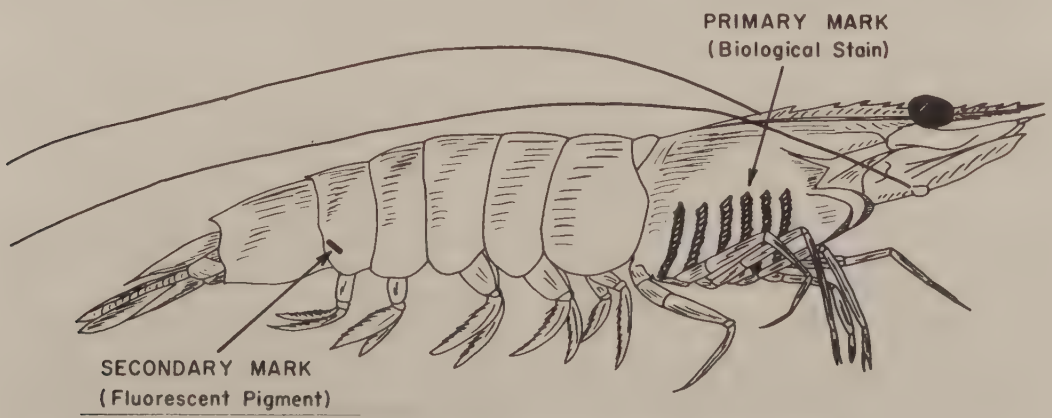


Figure 1.--Location of marking materials in double-marked shrimp.

Table 3.--Results of experiments to determine the suitability of checkwriter inks used with fast green FCF

[Determination of the condition and location of secondary marks was made after death. Results from shrimp that died within 10 days are not included]

| Experiment | Secondary mark | | | Length of experiment | Condition of secondary marks at injection site | | | Specimens which had ink in gills and at injection site |
|---|---|---|---|---|---|---|---|---|
| | Ink | Base | Dilution | | Good | Poor | Unsatisfactory | |
| | | | | Days | Number | | | Percent |
| 1..... | Red..... | Castor oil.. | 1:2 | 61 | 20 | 4 | 0 | 83 |
| | Blue.... | do..... | 1:2 | 61 | 25 | 2 | 0 | 100 |
| | Black... | do..... | 1:2 | 61 | 17 | 1 | 2 | 100 |
| 2..... | Red..... | None..... | None..... | 84 | 22 | 0 | 1 | 77 |
| | Blue.... | do..... | do..... | 84 | 16 | 0 | 0 | 69 |
| | Black... | do..... | do..... | 37 | 6 | 12 | 6 | 89 |

Table 4.--Summary of experiments involving fluorescent marks applied by the double-injection technique of marking

[Determination of the location of the secondary marks was made after death. Results do not include shrimp that died within 10 days of marking]

| Experiment | Primary mark | Secondary mark | | | Experimental shrimp | Length of experiment | Specimens which had fluorescent material in gills and at injection site |
|---|---|---|---|---|---|---|---|
| | | Pigment | Base | Dilution | | | |
| | | | | | Number | Days | Percent |
| 1..... | Fast green | Blaze orange A-15 | Castor oil | 1:50 | 50 | 61 | 100 |
| | do..... | Neon red A-12 | ....do..... | 1:50 | 50 | 61 | 100 |
| 2..... | do..... | Resoform yellow 10-2001. | Petroleum jelly | 1:25 | 50 | 84 | 52 |
| | do..... | Neon red A-12..... | ....do..... | 1:25 | 50 | 84 | 43 |
| | do..... | Blaze orange A-15..... | ....do..... | 1:25 | 50 | 84 | 53 |
| 3..... | do..... | Neon red A-12..... | ....do..... | 3:100 | 50 | 55 | 16 |
| | do..... | do..... | ....do..... | 3:100 | 50 | 72 | 36 |
| 4..... | Fast green | Neon red A-12..... | Petroleum jelly | 1:150 | 120 | 276 | 95 |
| | do..... | Blaze orange A-15..... | ....do..... | 1:150 | 120 | 276 | 82 |
| | do..... | Arc yellow A-16..... | ....do..... | 1:150 | 120 | 192 | 88 |
| | do..... | Saturn yellow A-17..... | ....do..... | 1:150 | 120 | 188 | 85 |
| | do..... | Resoform yellow 10-2001 | ....do..... | 1:150 | 120 | 276 | 93 |
| 5..... | do..... | Saturn yellow A-17..... | ....do..... | 1:150 | 78 | 24 | 2 |
| 6..... | do..... | do..... | ....do..... | 1:150 | 78 | 48 | 12 |
| 7..... | Trypan blue | do..... | ....do..... | 1:150 | 78 | 56 | 2 |

pigments appear to provide marks that meet the requirements of field mark-recapture studies.

## Single-Injection Method of Marking

This method consists of injecting a mixture of primary and secondary marking materials in a single operation. Various combinations of fast green and the fluorescent solvent, Fluorographic, were tested as combination marks. The mixture of these materials appeared green in color under white light and chalk white under ultraviolet light. After injection, the fast green concentrated in the gills within 24 hours, whereas the fluorescent solvent remained diffused throughout the body for 2 days and then began to concentrate in the gills. Mixtures of 0.05 percent fast green and Fluorographic in ratios of 7:1 or 8:1 produced

Table 5.--Comparative survival of shrimp receiving no mark, injected with distilled water, or marked with a mixture of two dyes

| Days | Material | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Experiment 5 | | | Experiment 6 | | | Experiment 7 | | |
| | None | Distilled water | Fast green and Saturn yellow | None | Distilled water | Fast green and Saturn yellow | None | Distilled water | Trypan blue and Saturn yellow |
| | Number | Number | Number | Number | Number | Number | Number | Number | Number |
| 0... | 78 | 78 | 78 | 78 | 78 | 78 | 78 | 78 | 78 |
| 1... | 72 | 76 | 78 | 78 | 78 | 78 | 77 | 68 | 67 |
| 2... | 68 | 73 | 73 | 76 | 78 | 78 | 73 | 66 | 64 |
| 4... | 67 | 72 | 68 | 76 | 78 | 78 | 71 | 63 | 62 |
| 8... | 65 | 71 | 65 | 76 | 78 | 78 | 68 | 62 | 59 |
| 12... | 57 | 67 | 57 | 76 | 77 | 77 | 64 | 61 | 55 |
| 16... | 54 | 60 | 53 | 75 | 76 | 77 | 56 | 57 | 54 |
| 20... | 45 | 44 | 41 | 74 | 75 | 77 | 39 | 49 | 47 |
| 24... | 32 | 34 | 36 | 66 | 69 | 74 | 37 | 44 | 42 |
| 28... | - | - | - | 62 | 68 | 73 | 30 | 39 | 40 |
| 32... | - | - | - | 61 | 67 | 72 | 25 | 36 | 34 |
| 36... | - | - | - | 61 | 65 | 66 | 23 | 33 | 31 |
| 40... | - | - | - | 54 | 58 | 60 | 15 | 26 | 26 |
| 44... | - | - | - | 46 | 48 | 58 | 13 | 18 | 19 |
| 48... | - | - | - | 41 | 27 | 44 | 8 | 15 | 12 |
| 52... | - | - | - | - | - | - | 5 | 11 | 12 |
| 56... | - | - | - | - | - | - | 3 | 5 | 8 |

suitable marks. Higher concentrations of the fluorescent solvent tended to mask the green dye. The fluorescent secondary mark could be detected under ultraviolet light for up to 45 days after marking, but soon thereafter began to fade. Consequently, this combination mark is suitable only for short-term studies.

### Tattooing

A secondary mark of ink or fluorescent pigment was also applied by tattooing in the manner described by Dunstan and Bostick (1956). A 1-percent suspension of a fluorescent pigment in castor oil, or one part Sanford's ink to three parts castor oil, was tattooed into shrimp between the second and third abdominal segments. Within a short time, these secondary marks were lost and an infection was observed at the injection site; therefore, this method of marking is not suitable for shrimp.

## SUMMARY

Prior to this study, fast green FCF, Niagara sky blue 6B, and Trypan blue were the only stains proved suitable for marking shrimp in studies of growth and mortality. Laboratory experiments were therefore made to find additional materials that could be used as shrimp-marking agents.

None of the 65 staining materials tested was found to provide fully satisfactory primary marks. Many of the stains were either lethal or faded within 1 week. Combinations of Trypan red and Trypan blue produced marks in the gills that could only be identified by trained observers and would therefore have limited practical use. Green and blue machine inks produced marks that faded but could still be used in short-term marking studies designed to provide general information on shrimp movements.

Various inks and fluorescent materials were tested for use as secondary marks in combination with the suitable biological stains. Red and blue checkwriter inks were found to be satisfactory for possible use as secondary marks in field studies designed to obtain general information on movements. Although the inks remained visible up to 84 days after marking, they frequently migrated into the gills and masked the primary stain. A combination of fast green FCF and the fluorescent solvent Fluorographic produced a combination mark adequate for short-term studies.

Five fluorescent pigments provided suitable secondary marks which could be used with fast green as a combination mark in experiments designed to estimate rates of growth and mortality. It was possible to localize the fluorescent material in two areas of the shrimp's body, thereby increasing the number of combination marks for shrimp. These marks were permanent and did not affect survival.

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